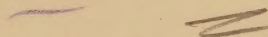


HISTORY OF THE GERM CELLS IN SPHAERIUM STRIATINUM (LAM.)

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FIFTY-FOUR FIGURES

AUTHOR'S ABSTRACT

Study of living and sectioned material throughout the life cycle shows the germ-cell history from fertilized egg to sexual maturity. This can be divided into the following five periods with definite limits: Original appearance during cleavage, period of inactivity, period of multiplication, maturation, and fertilization. Primordial germ cells of characteristic structure can be recognized just before gastrulation, when there is one large germ cell in the mass of mesoderm on either side of the blastocoel. After one division in each of these two cells, the four daughter cells remain inactive, while the remainder of the mesoderm differentiates, until division is resumed in the developing gonad. An indefinite number of gametes is produced. All are direct descendants of the two original primordial germ cells. Transformation of somatic cells into germ cells does not occur nor do germ cells become somatic cells. Cell lineage shows the two primordial germ cells to be derived from the third division of the paired mesoderm cells which have arisen by an equal division of the fourth micromere produced by cell D of the four-cell stage. Details of meiosis have not been ascertained, because of the small size of the chromosomes, their large number, and the difficulty of fixation. Nothing forecasts which primordial germ cells will become ova and which spermatozoa.

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INTRODUCTION

The problem which is the subject of this investigation was brought to my attention in the summer of 1921 during a study of the embryology of *Sphaerium striatinum*. In this study it became evident that the germ-cell history had not been adequately described in any species of the family Cyrenidae, although the literature contained material regarding certain phases of the subject. It therefore seemed desirable to describe the history of these cells more completely, to relate the scattered observations of previous workers, and to make comparisons with other Mollusca. The work was first concerned with the early history of the germ cells as shown by cell lineage. Later an attempt was made to determine the details of meiosis. It was hoped that differences might be found in the primordial germ cells that would forecast later differentiation into ova or spermatozoa, thus permitting a description of the complete history of the germ cells in a hermaphroditic mollusc.

In this connection, I wish to express my appreciation of the assistance received throughout the work from Prof. W. C. Curtis, at whose suggestion the problem was undertaken. My thanks are also due Prof. M. J. Guthrie for advice regarding cytological methods and to the founders of the Jonas Viles, Jr., Scholarship, which enabled me to work at the Marine Biological Laboratory at Woods Hole, Massachusetts.

MATERIAL AND METHODS

The species studied was *Sphaerium striatinum*, although comparisons were made with *S. ferrisi*, with one undetermined species of *Sphaerium*, and with three species of *Musculium*. Dr. Bryant Walker, honorary curator of the Department of Molluscs of the Museum of Zoölogy, University of Michigan, verified my identification of material collected near Columbia, Missouri; and Dr. W. J. Clench, curator of molluscs, Museum of Comparative Zoölogy, Harvard University, verified the identification of material collected at Woods Hole, Massachusetts.

All members of the families Cyrenidae and Sphaeridae are hermaphroditic. In *Sphaerium striatinum* the genital glands have a superficial position in each side of the body extending from the liver to the nephridium. The anterior portion of each gonad is lobed and constitutes the male portion. In large specimens this connects with the single enlarged female region by a short duct. In small specimens the male and female regions are not so distinctly separated. The wall of a single cavity may show maturing spermatocytes at one end and growing oocytes at the other, thus constituting a true ovotestis, while other lobes are definitely either male or female. The female portion of the gland is continued posteriorly as the hermaphroditic duct, which empties into the cloacal chamber near the opening of the excurrent duct from the kidney.

All the stages of development occur in brood pouches, which are closed sacs developed from the lamellae of the inner gills. The formation of these brood pouches and the nutrition of the embryos have been described by Poyarkoff ('01), Schereschewsky ('11), and Groenewegen ('26). In correlation with this development within a brood pouch the trochophore and later larval stages, which are conspicuous in pelecypods having a pelagic mode of development, are reduced in *Sphaerium*. Reproduction seems to occur throughout the year. Embryos as well as maturing germ cells are found in specimens taken in all seasons, although sperm and ova are not so numerous in winter.

Material was collected chiefly from the vegetation and mud bottoms of ponds in the vicinity of Columbia, Missouri. For comparison I had specimens obtained at Woods Hole and about one hundred slides from Doctor Curtis' collection.

Fixation

In the study of cell lineage whole specimens, gills containing brood pouches, and embryos of various ages dissected from the brood pouches were fixed in either hot or cold Bouin's fluid, Zenker's fluid, Zenker's with formic instead of

acetic acid (compare Guthrie, '26), and corrosive-sublimate-acetic acid. Other fixing fluids have since proved equally satisfactory for the histological features.

In attempting to secure material that would permit counting the chromosomes and observations on the nuclear changes of meiosis, whole animals, embryos, gills, and smears of fresh tissue were fixed in Allen's fluid, Bouin's fluid with formic instead of acetic acid, Carnoy's, Carnoy's with formic instead of glacial acetic acid, the fluid of Carnoy and Lebrun, Flemming's fluid, Zenker's fluid with copper sulphate instead of sodium sulphate, and a fixative used by Doctor Guthrie. This last consists of 15.0 cc. of saturated aqueous solution of picric acid, 1.0 cc. of 3 per cent chromic acid, and 1.0 cc. of formic acid. All these fluids were used at room temperature. Specimens were also teased apart in pond water under a binocular, in tap-water, distilled water, 0.2, 0.6, and 0.9 per cent sodium-chloride solution, and the small pieces thus obtained were transferred to each of the fixing fluids listed above. Such preparations showed no better fixation of chromosomes than whole specimens fixed in the same fluids.

Staining

The material was embedded in paraffin, and sectioned 4, 5, 7, 8, and 10 μ , depending on the features to be studied. Heidenhain's iron haematoxylin and Mayer's haemalum were used with eosin or alkaline orange G in 95 per cent alcohol as counterstain. Such preparations were satisfactory for the general histological features. For observations upon the nucleus the rapid method of using Regaud's haematoxylin gave as good results as the longer method of using Heidenhain's iron haematoxylin. Ehrlich's acid haematoxylin and toluidin blue were also tried. No combination of fixation and staining gave clear chromosome pictures even when sections were bleached with hydrogen peroxide before staining.

Other methods

Embryos in very early gastrula stages and all later stages were dissected from the gills under a binocular microscope and placed in water on a slide for study under higher magnification in both transmitted and reflected light. Camera-lucida drawings of the cleavage and later stages in serial sections were made on transparent paper, and clay models were then constructed, from which figures of the cleavage stages and embryos could be drawn as though from the entire object. For the cleavage stages two drawings of each section were made on the same sheet of transparent paper: one with the upper surface of the section in focus, drawn in red; the other with the lower surface in focus, drawn in black. These drawings were superimposed and models constructed in clay. This method proved satisfactory and required less time than the use of wax plates. In making these models the relative size and position of the cells were carefully followed without attempting to reproduce unimportant details in the actual shape of the cells.

OBSERVATIONS AND INTERPRETATIONS

The primordial germ cells are first recognized as two large cells (fig. 32, *G* and *G.1*), one on either side of the embryo in the early gastrula stage. They lie in contact with a mass of other mesodermal cells on either side of the invaginating endoderm. Meisenheimer ('01 a) describes this production of primordial germ cells as the first differentiation in the mass of cells destined also to give rise to the nephridia, heart, and pericardium. Since the appearance of cells that can be easily recognized as primordial germ cells is thus delayed, their earlier history can be determined only by ascertaining the cell lineage of these two masses of mesoderm cells in which the two primordial germ cells first appear. The work of Whitman ('78 and '87), Wilson ('89 and '92), Conklin ('97), and many others show that cell lineage provides the only means of forecasting the earliest stages of differentiation if distinctive features are not present in cells from which certain organs develop.

Fertilization

The method and time of fertilization of the egg in *Sphaerium* are not known. I have found no unfertilized eggs in the gills and have found only two fertilized eggs in a stage before the formation of the first cleavage spindle. This confirms the observations of Stauffacher ('93).

Repeated attempts to keep isolated individuals in aquaria to determine whether self-fertilization occurs have been unsuccessful. The animals die within four months. Both ova and sperm are present at the same time in mature individuals. The sperm pass through the ovarian region of the gonad to the outside. Figure 3 shows a spermatozoon at the micropyle of an egg in the hermaphroditic duct, possibly indicating that fertilization occurs there, although such a relationship between sperm and ovum might be accidental, since both pass through this duct to the outside. Ziegler ('85) and Gilmore ('17) state that self-fertilization probably occurs, but offer no evidence other than the anatomical structure of the reproductive organs. Self-fertilization has been demonstrated in some of the hermaphroditic molluscs by Colton ('12 and '22) and Crabb ('27 a and b). This may be the case in *Sphaerium*, but there is no positive evidence. From existing evidence it is not known whether self-fertilization occurs in *Sphaerium*.

Cleavage

One to four cells. In designating the cells of the cleavage stages, the following terminology is used: The first four cells are assigned capital letters, *A*, *B*, *C*, and *D*. These are the macromeres. The generations of micromeres are designated by small letters *a.1*, *b.1*, *c.1*, *d.1*, and *a.2*, *b.2.1*, *c.2.2.1*, etc. The first number following the letter is the generation of micromeres from which the cell was derived. In addition to this, the cell receives other numbers indicating the generations of cells that have occurred since the micromere was cut off from the macromere. Thus: $a.1.1 < \frac{a.1.1.1}{a.1.1.2}$ shows that the cell *a.1.1* is a descendant of the first generation of micromeres arising from *A*, that it is the result of the first division

EXPLANATION OF FIGURES

Figures 4 and 5 and figures 15 to 20 were made from models prepared from serial sections drawn in outline under the microscope. Figures of sections were made with a camera lucida to represent as nearly as possible the appearance of the material on the slide. The magnification after reduction in reproducing the figures is indicated for each figure.

ABBREVIATIONS FOR ALL FIGURES

<i>a.1, b.1, c.1, etc.</i> , first generation of micromeres	<i>M</i> , mesoderm-building cell
<i>a.2, b.2, c.2, etc.</i> , second generation of micromeres	<i>M.1, M.1.2, m.2, m.2.2.1, etc.</i> , descendants of <i>M</i>
<i>a.3, b.3, c.3, etc.</i> , third generation of micromeres	<i>mch</i> , mesenchyme of larval mesoblast
<i>a.2.1, b.3.1, a.1.1, etc.</i> , descendants of the generation of micromeres indicated by the numeral after the latter (compare p. 550)	<i>mes</i> , mesoderm
<i>A, B, C, D</i> , macromeres of the four-cell stage	<i>mit</i> , mitochondria
<i>aam</i> , anterior adductor muscle	<i>ml</i> , mantle line
<i>bg</i> , rudiment of the byssus gland	<i>n</i> , nephridium
<i>cd</i> , cavity of the hermaphroditic duct	<i>nu</i> , nucleus
<i>cg</i> , cerebral ganglion	<i>nuc</i> , nucleolus
<i>d.4 (M)</i> , mesoderm-building cell	<i>oes</i> , oesophagus
<i>ect</i> , ectoderm	<i>oog</i> , oogonia
<i>em</i> , egg membrane	<i>ot</i> , oocyte
<i>end</i> , endoderm	<i>ovc</i> , cavity of the ovary
<i>ent</i> , enteron	<i>p</i> , pericardium
<i>es</i> , excurrent siphon	<i>pam</i> , posterior adductor muscle
<i>f</i> , foot	<i>pb</i> , polar body
<i>G, G.1</i> , first two germ cells	<i>per</i> , peritoneum
<i>g</i> , gonad	<i>n per</i> , nucleus of peritoneal cell
<i>gc</i> , germ cells	<i>pg</i> , pedal ganglion
<i>ggo</i> , cavity of gonad	<i>R</i> , rectum
<i>gi</i> , gill	<i>S</i> , shell
<i>gr</i> , granules (mitochondria)	<i>sc</i> , segmentation cavity
<i>h</i> , heart	<i>sdt</i> , spermatid
<i>hpn</i> , anlage of heart, pericardium, and nephridium	<i>sd</i> , sperm duct
<i>hp</i> , anlage of heart and pericardium	<i>sg</i> , shell gland
<i>is</i> , incurrent siphon	<i>spe</i> , spermatocyte
<i>L</i> , liver	<i>spg</i> , spermatogonia
<i>lp</i> , lower pericardial cavity	<i>spz</i> , spermatozoa
	<i>st</i> , stomach
	<i>t</i> , testis
	<i>tc</i> , cavity of testis
	<i>vg</i> , visceral ganglion
	<i>wd</i> , wall of hermaphroditic duct
	<i>4c</i> , four chromosomes that precede the others in the anaphase

of this micromere, and that it divided into two cells each of which carries with it the numbers of the parent cell with the addition of another number. The only exceptions to this terminology are the cell *d.4*, which is designated as *M*, since it is a mesoderm-forming cell, and the large cell remaining after the third generation of cells following the bilateral division of *M* which are designated as *G* and *G.1*, since they are germ cells (compare tables 1 and 2). This terminology is similar to that used by Wilson ('92) for *Nereis*, Lillie ('95) for *Unio*, and Tannreuther ('15) for *Bdellodrilus*.

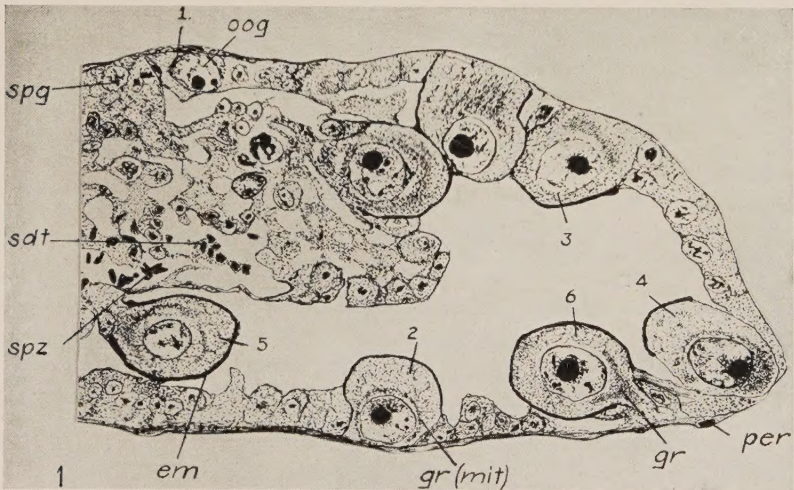


Fig. 1 Longitudinal section through a maturing gonad. Underlined numerals indicate successive stages in the growth of the oocytes. $\times 290$.

Since this study deals with the germ cells, the cleavage in other regions was followed only so far as was necessary to determine the absence of any contribution to the formation of germ cells. The more general comparisons will not be made, since this has been done by Wilson ('92), Lillie ('95), Conklin ('97), Tannreuther ('15), and others. References to the literature will be made only as seems necessary in making my own account clear.

The ovum of *Sphaerium*, which is slightly oval, is oriented from the earliest stages (fig. 2). A membrane develops around the egg while it is still attached to the wall of the ovary (fig. 1, *em*). The point of attachment is marked by a large micropyle. A shift of cytoplasmic materials is indicated by the fact that the mitochondrial cloud, which is adjacent to the micropyle in the attached egg (fig. 1, no. 6, *gr.*), lies to one side of the micropyle in eggs free in the ovary

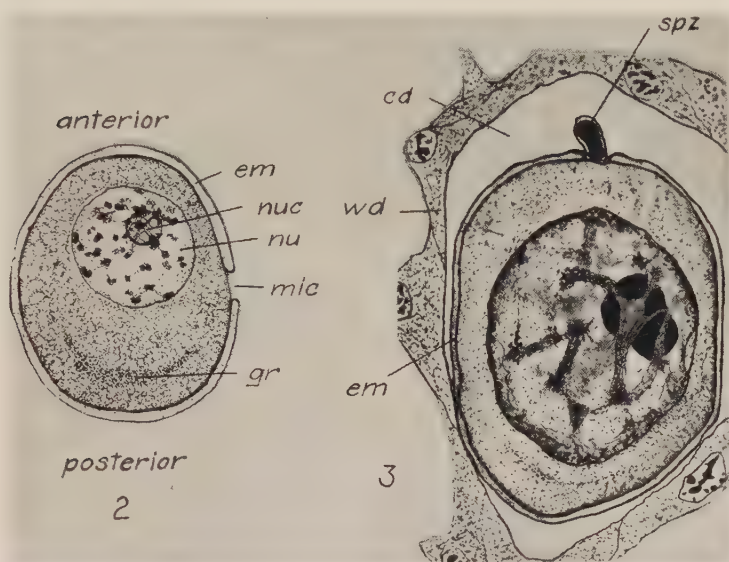


Fig. 2 Diagram showing orientation of the egg before fertilization.

Fig. 3 Sperm and ovum in the hermaphroditic duct. $\times 900$.

(compare fig. 1, no. 6, and fig. 2). The nucleus shifts its position from the side of the egg opposite the micropyle to a place nearer the micropyle, but not adjacent to it. A line drawn through the nucleus and through the thickest and thinnest regions of the cytoplasm lying on opposite sides of the nucleus represents the future anteroposterior axis. Mitochondria seem to be the only storage material in the cytoplasm. A detailed account of the origin, distribution through cleavage, and significance of these mitochondria will appear

in another paper. The position of the nucleus, the micropyle, and the cloud of mitochondria establish the orientation of the egg with reference to the anteroposterior axis of the embryo. Dorsoventral orientation is not established until the second cleavage. Segmentation is holoblastic and unequal from the beginning.

The first cleavage spindle lies in the long axis of the egg and is nearer the animal pole. As a result, the egg divides into two cells of unequal size, *AB* and *CD*, at about the level of the polar bodies (figs. 6 and 15). Before the first cleavage

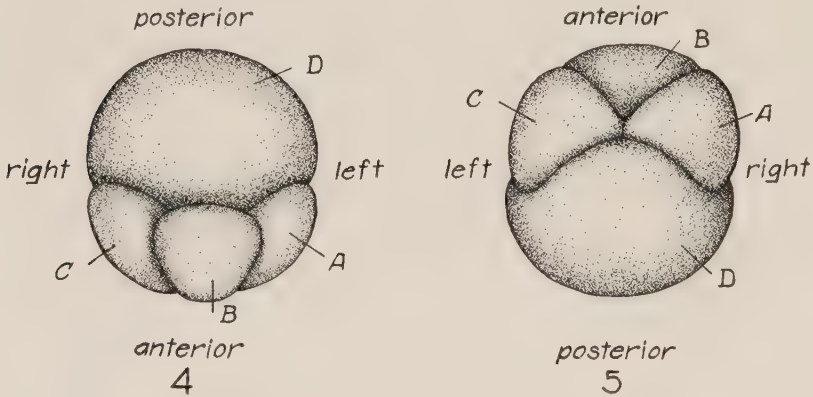


Fig. 4 Orientation of the four-cell stage as seen from above.

Fig. 5 Orientation of the four-cell stage as seen from below.

spindle forms, the chromosomes appear and the large vesiculated nucleolus becomes diffuse. At the same time, a clear area appears in the cytoplasm around the posterior side of the nucleus. When the nuclear membrane disintegrates and the spindle is formed, the nucleolus is no longer visible, but it reappears when the nucleus is reorganized in the daughter cells. The nuclei in *AB* and *CD* are spherical. The cytoplasm of *CD* is dense and finely granular, while that of *AB* is more hyaline. The cell membranes are distinct and the cells lie flattened together within the egg membrane (fig. 6). The two cells taken together approximate the shape of the mature egg.

The second cleavage is almost at right angles to the first and is well advanced in *AB* before there is any indication of spindle formation in *CD*. The result is a three-cell stage consisting of two small macromeres, *A* and *B*, of equal size, slightly to the left of the future midline of the body, and the large cell *CD*. The contact surfaces are flattened as they were in the two-cell stage (figs. 7 and 16).

After cleavage has been completed in *AB*, constriction occurs in *CD* and the four-cell stage results. The spindle is tilted (fig. 8) so that the cell *C* occupies a position on the cell *D*, as indicated in figure 17. On the future dorsal surface (fig. 4), the cells *B* and *D* are in contact, while *A* and *C* (fig. 5) are in contact for a shorter distance on the ventral surface. As seems to be the case in all eggs in which the greater mass of the four cells forms the ectoderm, the cross furrow resulting from this arrangement (fig. 4) is longer at the animal pole. In forms like *Clepsine* and *Nereis* the greater mass of the four cells becomes endoderm and the furrow is longer at the vegetal pole. Unlike the case of an egg having free development, the cross furrow is of little value as a landmark for orientation in *Sphaerium*.

Exact orientation of the four-cell stage is important for determination of the origin of regions that later develop (figs. 4 and 5). The cell *B* is anterior, *C* is on the future right side, *A* is on the left, and *D* is posterior. These cells are the future posterior ventral surface of the embryo, while the region of micromere production is the future dorsal surface. The future median plane passes through the cells *B* and *D*.

The cytoplasmic structure of the four-cell stage is much the same as in the preceding stages. The large cell *D* (fig. 9) is more nearly opaque and its cytoplasm more densely granular than the cytoplasm of the other three cells. The nucleus of *D* is slightly larger than the nucleus of *C*, which in turn is larger than the other two nuclei. The first indication of a blastula cavity appears in the four-cell stage (fig. 9).

First generation of micromeres. The production of micromeres occurs on the upper surface of the macromeres in a

somewhat irregular manner. After *C* has been cut off from *D*, it divides to produce *C* and *c.1*, thus forming a five-cell stage (fig. 18). This *c.1* is the first cell of the first generation of micromeres. Division in *C* is oblique. The micromere end of the spindle is uppermost and inclined toward the right, so that *c.1* lies between *C* and *D* on the upper surface. There are now four cells on the anterior upper surface of *D*. The second micromere of the first generation is produced by *D*. The spindle is tilted toward the right as it was in *C*, and as a result *d.1* lies on the surface in contact with *B*, *D*, *c.1*, and *D*. Cleavage in *D* usually begins before spindles form in *A* and *B*. *A* and *B* divide at about the same time and may complete their division to produce *a.1* and *b.1*, respectively, shortly after *d.1* is completely separated. The result is an eight-cell stage (fig. 21). Usually *d.1* has begun to divide before *a.1* and *b.1* have been cut off completely, so that a nine-cell stage results with the separation of *d.1* into *d.1.1* and *d.1.2*. The spindles in both *A* and *B* are inclined clockwise. The cell *a.1* lies between *A* and *B* and the cell *b.1* lies between *B* and *C* (fig. 23).

The internal structure of the micromeres is similar to that of the cells *A*, *B*, and *C*. The cytoplasm of the cell *D* is more coarsely granular and lacks the reticular appearance that is seen in the other cells after fixation. The nucleus in *D* is larger than those of the smaller cells. The egg membrane, which has been visible up to this time as a homogeneous cov-

Fig. 6 Section of the two-cell stage. $\times 430$.

Fig. 7 Section of a three-cell stage. $\times 430$.

Fig. 8 Reconstruction from three sections, showing the spindle that produces *C* and *D*. $\times 400$.

Fig. 9 Section of a five-cell stage between the lines shown in figure 18. $\times 430$.

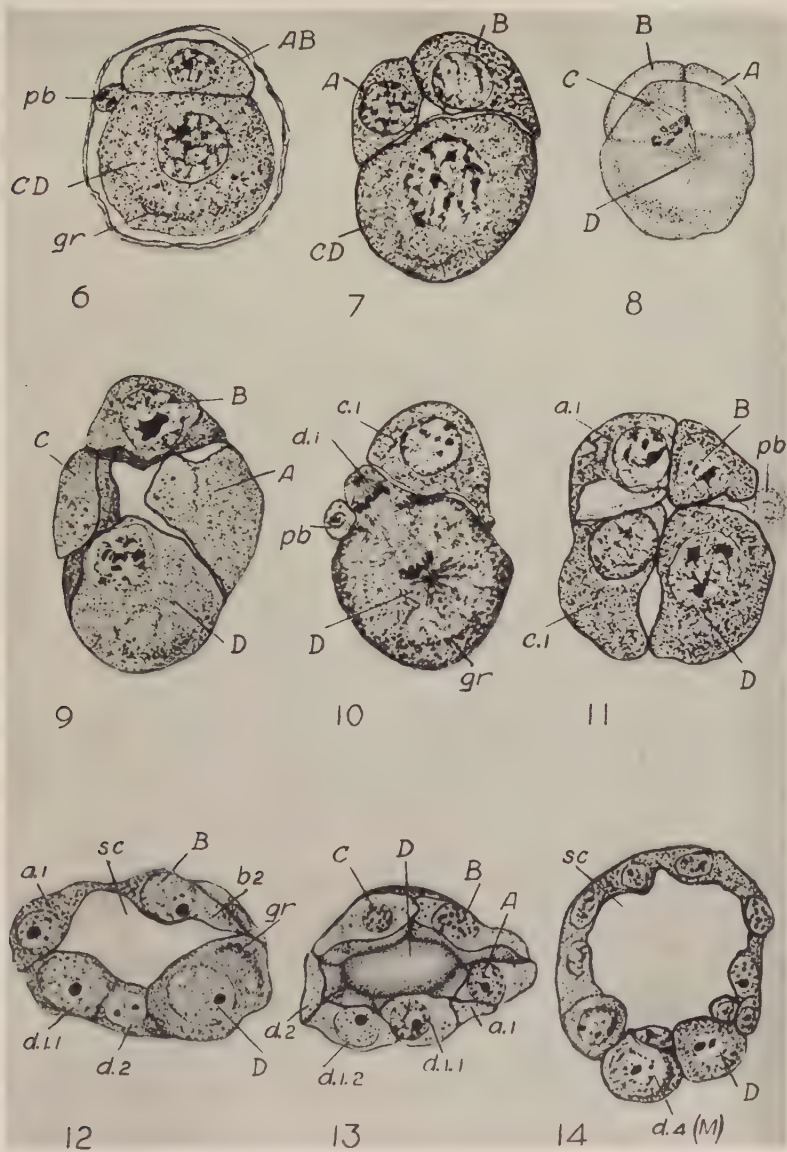
Fig. 10 Section through a five-cell stage, showing the spindle that produces *d.1*. $\times 430$.

Fig. 11 Section of an eight-cell stage. $\times 430$.

Fig. 12 Section of a twelve-cell stage at level 12 of figure 22. To show relative size of cells and segmentation cavity. $\times 430$.

Fig. 13 Section at level 13 of figure 23. Half-schematic figure constructed from three sections. $\times 430$.

Fig. 14 Section of thirty-two-cell stage, showing *D* and *M* on the surface before migration of *M* to the inside. $\times 430$.



Figures 6 to 14

ering around the dividing cells, now begins to disappear. The blastula cavity is now more evident and lies within the upper half of the mass of cells. The unequal cleavage just described can be correlated with the fact that *CD* gives rise to cells that later produce the shell gland. The larger size of cell *D* can be correlated with the fact that it contains most of the future mesoderm and some of the endoderm. The cleavage is oblique, since the spindle in each of the macromeres inclines so that the micromeres are located to the right and above the cells that produce them.

Second and third generations of micromeres. Inequalities in the division rate in various regions result in some irregularity in the location of cells. This obscures the symmetry that is so clearly shown in the cleavage stages of animals that produce micromeres synchronously. While the division of *d.1* to form *d.1.1* and *d.1.2* progresses, the cell *D* gives off *d.2*, which is the first of the second generation of micromeres. Thus a ten-cell stage is formed (fig. 23). Division in *A* occasionally precedes division in *B*, and occurs at the time that *d.2* is being produced, so that an eleven-cell stage occurs.

The usual arrangement of cells, as shown in figure 21, is followed by cleavage in *A*, *B*, and *C*, forming a thirteen-cell stage (fig. 24). Division in *A* and *B* is always slightly in advance of that in *C*, thus producing a twelve-cell stage (fig. 22). The order of production of the second generation of micromeres is *d.2*, *a.2*, *b.2*, and *c.2*. The cell *d.2* is homologous

Fig. 15 Model of two-cell stage.

Fig. 16 Model of the three-cell stage. Position of the spindle shown in *D*.

Fig. 17 Model of the four-cell stage from the lower pole.

Fig. 18 Model of the five-cell stage from the upper pole.

Fig. 19 Model of the six-cell stage from the upper pole and slightly to the left.

Fig. 20 Model of a seven-cell stage, a case in which division of *d.1* occurs before division occurs in *A* and *B*.

Fig. 21 Model of the eight-cell stage from above and posterior. Cell *B* is not visible.

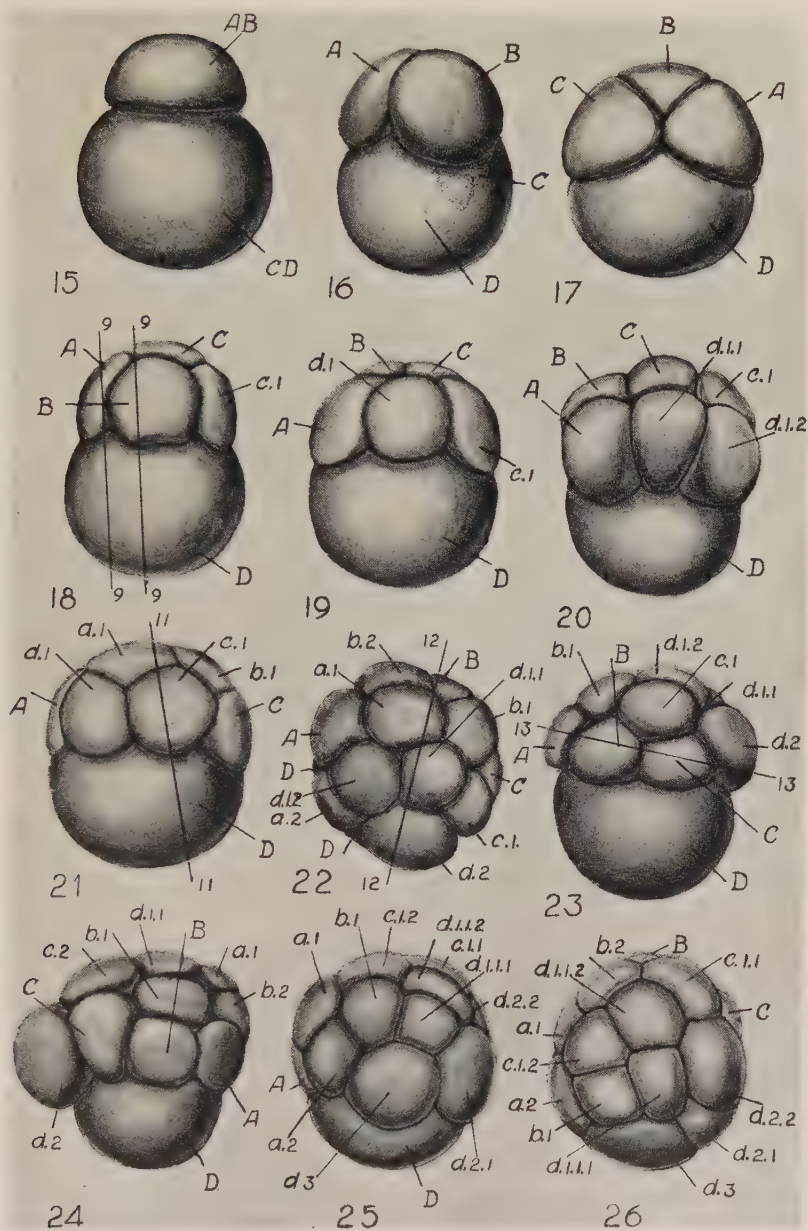
Fig. 22 Model of the twelve-cell stage from the upper pole.

Fig. 23 Model of the ten-cell stage from the side.

Fig. 24 Model of the thirteen-cell stage from the side.

Fig. 25 Model of the seventeen-cell stage from the side.

Fig. 26 Model of the seventeen-cell stage from the anterior end.



Figures 15 to 26

with the cell X of *Bdellodrilus* (Tannreuther, '15), two species of *Nereis* (Wilson, '92), *Clepsine* (Whitman, '78), and no doubt many other forms. Later it divides equally and gives rise, in all the forms in which its history has been followed, to the greater part of the ectoderm in the middle region of the embryo on the ventral side. I have not followed the cleavage of *d.2* past its third division, but, so far as I can determine, there is no connection between this cell and any portion of the mesoderm-forming cells. In addition to the cells resulting from the division of *d.2*, the cells produced by the subsequent divisions of *A* and *B*, or the cells *a.2* and *b.2*, contribute to the formation of the ectoderm, but in the anterior region of the body. Cells *a.2* and *b.2* probably give rise to certain cells that become the larval mesoblasts. These are designated by Ziegler ('85) as mesenchyme cells and later form the single-celled muscle fibers of the embryo. Their exact origin has not been determined. It seems certain that they have an entirely different origin from the principal mass of the mesoderm, because they are present before the definitive mesoderm masses appear in the blastula cavity. The formation of larval muscles from the descendant of 'Y' (or *a.2.2.1*) has been described in *Unio* by Lillie ('95). Stauffacher ('93) recognized these cells as ectodermal in origin and so confirmed the supposition of Ziegler ('85) that the mesenchyme cells are derived from ectoderm. This has also been described in other forms (compare Meisenheimer, '01 b).

The appearance of the nuclei and the relative size and position of the cells and segmentation cavity in the twelve-cell stage can be seen in figures 12 and 13. After the thirteen-cell stage, cleavage occurs in *d.2*, *d.1.1*, and *c.1*, while *D* gives rise to a third micromere, *d.3*, which is the largest so far produced. Although the cleavage of *d.3* was not followed past one division, it seems to lie in the region where the shell gland appears in later embryonic stages. Conklin ('97) finds that *d.2* gives rise to the shell gland in *Crepidula*. This is also true for *Unio*. It is possible I have misinterpreted its origin in *Sphaerium*, although the conclusion I have reached

seems to be justified by my observations. The result of these cleavages is a seventeen-cell stage (fig. 25). Cleavage in *C* results in the cell *c.2* being given off to the left, and *d.3* is given off to the right, opposite to the direction of *d.2*.

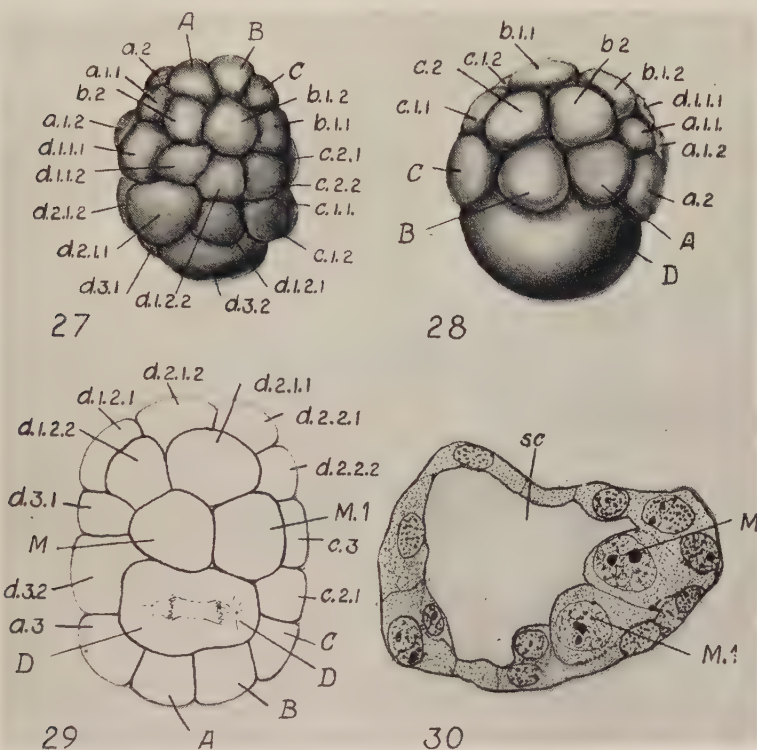


Fig. 27 Model of the twenty-four-cell stage from the upper pole.

Fig. 28 Model of the seventeen-cell stage from the upper pole.

Fig. 29 Diagram of the arrangement of cells about the posterior end just after division of $d4(M)$.

Fig. 30 Cross-section of a late blastula just after *M* and *M.1* have reached the interior. $\times 250$.

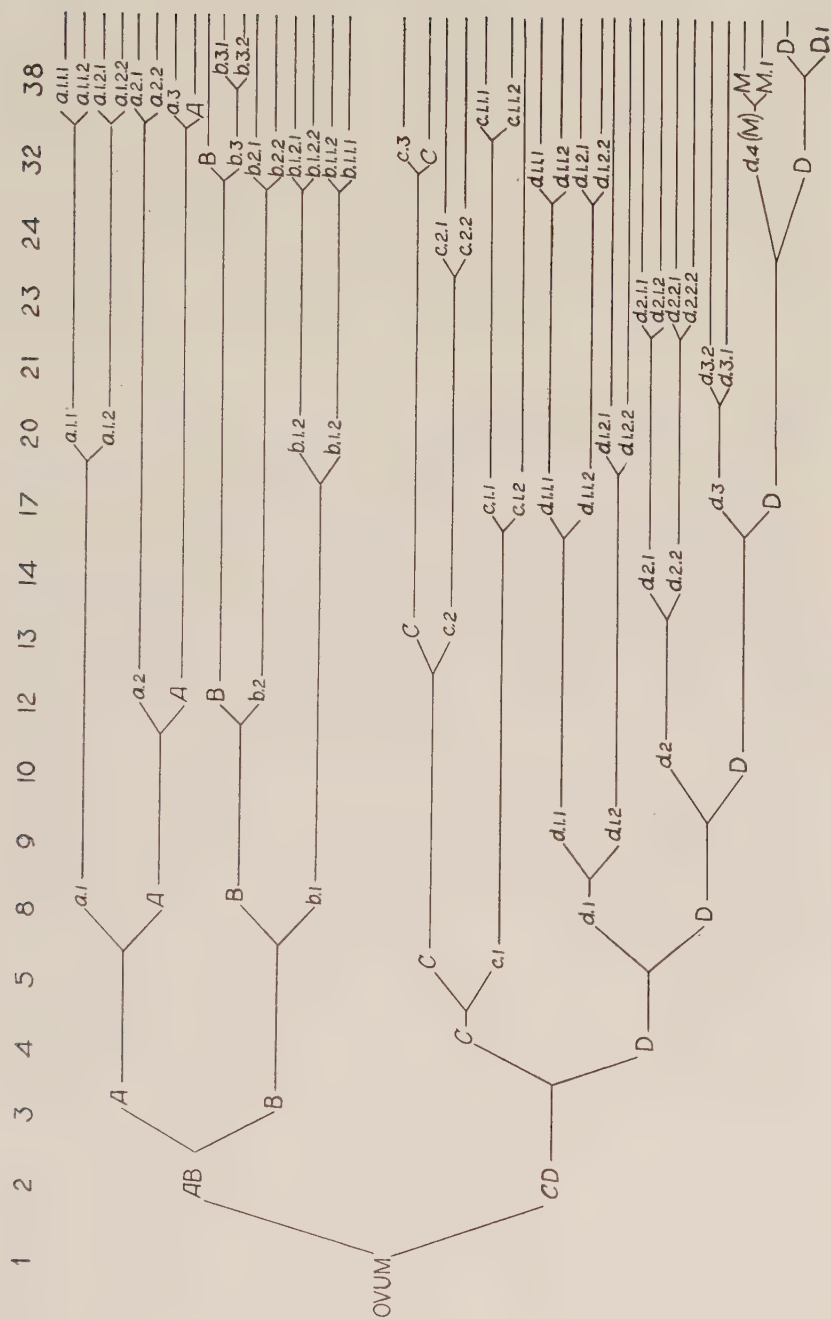
Cleavage in *a.1*, *b.1*, and *d.1.2* is followed by cleavage in *d.3* to produce a twenty-one-cell stage. Cleavage in *d.2.1*, *d.2.2*, and *c.2* produced a twenty-four-cell stage (fig. 27) before *b.3* and *c.3* are formed. The third generation of micromeres is given off to the right, opposite to the direction of the second generation.

Mesoderm formation and segregation. At about the twenty-four-cell stage *D* begins to divide equally to produce *d.4*, but the completion of this division is not accomplished until about the thirty-two cell stage. This stage, however, is merely a coincidence in the number of cells, since the smaller cells of the upper hemisphere have never divided synchronously. The fourth division of *D* is almost at right angles to the earliest division. With the increased number and changing position of the cells, there has also occurred an increase in the alternate change in the direction of the spindles in *D* until this last one is slightly more than 90° from the direction of the first cleavage spindle in the egg. The cell *d.4* (fig. 14), which is the mesoderm-forming cell, is now called *M*. From this time on the cell *D* contributes only to endoderm formation. Hence the production of *d.4* finally separates the mesodermal- and endodermal-forming capacities which were previously carried in *D*.

There is one equal division in *M*, forming *M* and *M.1* (fig. 29), and at about the same time there is an equal division in *D*, with the result that there are four large cells on the posterior surface of the embryo which are the products of the most recent divisions of *D*. In addition to these four cells, the cells *A*, *B*, and *C* on the posterior surface also form part of the endoderm. The number of cells present in the embryo at the time *d.4* is produced is somewhat variable in *Sphaerium* and differs greatly in different species. In *Bdellodrilus* it occurs at the twenty-four-cell stage, in *Unio* at the thirty-two-cell stage, in *Nereis* at the thirty-eight-cell stage, in *Dinophilus* at the forty-five-cell stage. In *Crepidula* *d.4* is produced at the twenty-nine-cell stage, although final separation of all the endoderm-forming material from *d.4* does not occur until the sixty-five-cell stage. The number of cells present when the primary mesoblast '*M*' (*d.4*) is produced cannot always be correlated with a precocious development in certain regions as was formerly thought (compare Lillie, '95, p. 77).

The statement of Stauffacher ('93) that the mesoderm is paired from the outset is true, if he means it is not mesoderm

TABLE 1



until it is on the inside. His statement may also be due to a different interpretation of the cells that lie in the region of the posterior end. According to my observations, the unpaired cell *M*, which lies on the surface of the embryo, is the parent of all mesodermal elements except the larval mesoblast. The macromere *D*, which is the cell *Ma.* in Stauffer's description, does divide into two parts as shown in figure 29, but the macromere *ma.2*, which he shows in his figure 28, is no doubt *M.1*, and the cell he labels as *ma.1* seems to be *M*. The cell he has labeled *um.1* in his figure 28 is the second division of *M*.

With the division of *M* to form *M.1* and *M*. (fig. 29), we find the paired mesoblasts on the posterior surface of the embryo adjacent to the cell *D*, which is dividing to form *D* and *D.1*. Both of these mesoderm-forming cells now begin to sink below the surface. Just before this migration each begins to bud off a smaller cell into the blastocoele. These new cells are on the inner surface of the parent cell, but lie in contact with the ectoderm when the division is completed. As the large cells *M* and *M.1* migrate inward, the cells on the surface divide and close the place that has been vacated (figs. 30 and 31). With the completion of this process there are four mesoderm cells, derived from the original unpaired cell *M* or *d.4*, lying within the cavity in contact with the posterior surface cells. There are also four cells on the surface that result from the two most recent divisions of *D*. The production of the smaller mesoderm cells is in such a direction that they lie next to the blastula wall. The division of the two smaller cells interposes another cell between the large cells *M* and *M.1* and the blastula wall. A second generation of small mesoderm cells is given off by *M* and *M.1.1.2*. These also lie next to the blastula wall and are the cells *m.2* and *m.1.2.1*. The result is a mass of small cells next to the blastula wall on either side, with one large cell on the inner surface of each mass. The cells *D* and *D.1* divide at about the same time as the first division of *M* and *m.1*. A third generation of small mesoderm cells is produced by *M* and *M.1.2.2*, form-

ing the cells *m.1.2.2.1* and *m.3*. This is the last unequal division of the primary mesoblasts. There are then two large cells, one on either side, and between these and the ectoderm are the smaller cells of similar origin. Since the smaller cells are produced between the larger cells and the blastula wall, they tend to push the large cells farther into the cavity. A few larval mesoblasts are also present (fig. 32, *mch.*), but they are of different origin from the two mesodermal masses

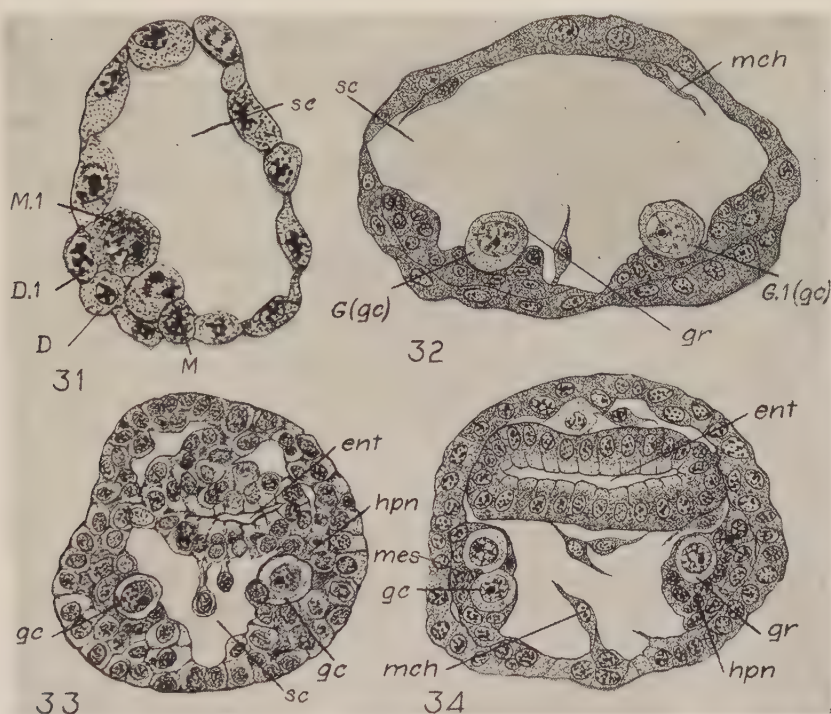


Fig. 31 Section through a blastula in which the migration of *M* and *M.1* to the interior is complete. $\times 215$.

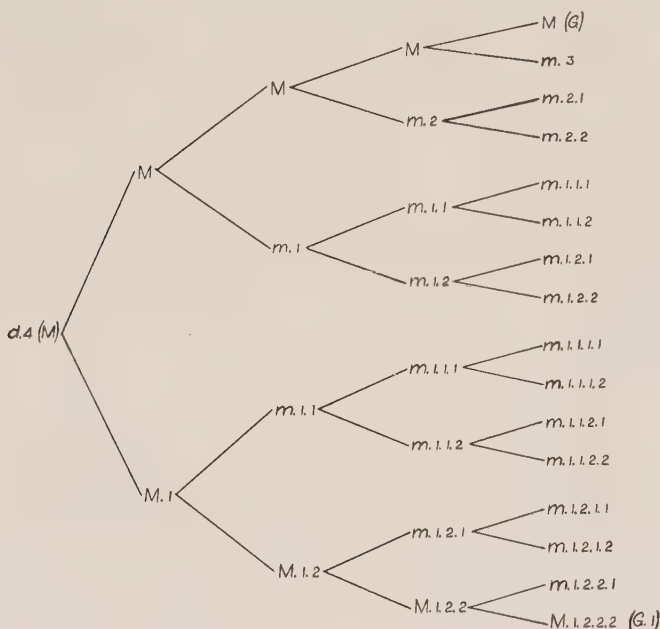
Fig. 32 Transverse section through a late blastula, showing the mesodermal masses on each side ventrally, and the two original primordial germ cells. $\times 300$.

Fig. 33 Transverse section through an early gastrula, showing the location of the germ cells and mesodermal masses. Only one germ cell shows on either side. $\times 250$.

Fig. 34 Transverse section through a late gastrula, showing location of germ cells. $\times 250$.

derived from the primary mesoblasts *M* and *M.1*. The large cells, *M* and *M.1.2.2.2* (*G* and *G.1* of fig. 32) may now be called germ cells, since they ultimately produce the definitive cells of the germ gland and retain their characteristics throughout the remainder of the embryonic development until they reach their place in the gonad. They are, moreover, the only cells that form the definitive germ cells and all the

TABLE 2



products of their subsequent divisions appear to be potential germ cells. Each of these cells *G* and *G.1* (fig. 32) divides once, equally, and at about the same time, along with the irregular division of other cells in the mesodermal masses. The daughter cells of *G* and *G.1* then remain inactive for a relatively long period. Stauffacher ('93), in his figure 32, shows a large cell in the mesoderm, as it appears on one side of the embryo, that has all the characteristics of a primordial germ cell, though he does not call it that.

In the mitoses that result in the setting aside of the primordial germ cells there is no evident difference in the nuclei of the larger and smaller cells except in size. Favorable material for the study of chromosomes has not been obtained. So far as I have been able to determine in *Sphaerium*, no elimination of chromatin occurs as in *Ascaris* (Boveri, '92) and *Miastor* (Kahle, '08, and Hegner, '12). Elimination of chromatin is not peculiar to primordial germ cells, as shown by Rhode ('11) and Wilson ('25). In *Sphaerium*, however, certain chromatin material always seems to go on the equatorial plate before the remainder. During the early anaphase (figs. 50 and 51) of mitosis there are also four of these masses ahead of the remainder of the chromatin in its passage toward the poles. I have no suggestion as to the reason for this. So far as I can determine, no chromatin is discarded in any mitosis.

During the migration of *M* and *M.1* the endoderm begins to be formed. The cell *D* divides equally and all its descendants become endoderm. Gastrulation occurs by a true invagination, as described by Lankester ('75 a and b, '76). At first the archenteron is short and extends into the segmentation cavity between the paired mesodermal masses and serves to separate them farther. Differentiation of the endoderm to a functional state, which has been described by Ziegler ('85) and others, need not be considered here. A comparison of the various cases in which the endoderm has different modes of origin is made by Tannreuther ('15). The amount of endoderm derived from *M* varies from cases like *Crepidula*, in which *M* gives rise to more endoderm than mesoderm, to the other extreme in which *M* contributes nothing to the endoderm, as in *Bdellodrilus*. In *Sphaerium*, also, *M* contributes nothing to the endoderm.

Discussion. Under normal conditions of development cleavage in *Sphaerium* is determinate. Both embryonic and adult structures are traceable from definite regions of the unsegmented egg. Orientation of the future embryo is partially forecast by the relative position of the nucleus, the

micropyle, and a cloud of granular mitochondria. Although cleavage thus separates the various regions of the egg having different capacities for differentiation, it is not believed that egg cytoplasm is passed unchanged through successive generations of cells. A detailed comparison of cleavage in the molluscs, annelids, and flatworms is not necessary in this discussion, but certain points must be considered.

The general plan of cleavage in *Sphaerium* is similar to that in many other forms. Three successive divisions separate the ectoderm from the basal cells. The fourth cleavage of the macromere *D* separates the mesoderm except for the larval mesoblasts. The remaining cells form the endoderm. In the molluscs, except the cephalopods and *Lymnaea*, in annelids, turbellaria, and nemertines, this mosaic development of the egg is of the oblique or spiral type with the first and third generations of micromeres arising dextrotropically and the second and fourth generations arising leiotropically, and this relationship is constant regardless of the wide differences in the size of the basal cells occurring in the various forms. It is not necessary to discuss the complicated problems concerned in the arrangement of cells during these cleavage stages except to point out that the rate of growth, size of the cells, their content and physical nature are all factors which are concerned in the positions that the cells occupy and in the form of the mass as a whole. Despite the development in a brood pouch, cleavage in *Sphaerium* is similar to the type commonly found in most of the annelids and molluscs that have been carefully studied.

That the germ cells are mesodermal in origin has long been known for *Sphaerium*, but their definite history through the cleavage stages has not been followed. The formation of the mesoderm from the fourth generation of micromeres arising from the cell *D* of the four-cell stage has been shown to occur in *Clepsine* (Whitman, '78); two species of *Nereis*, *Spio*, *Polymnia*, and *Aricia* (Wilson, '92); *Fiona* (Casteel, '04); *Amphitrite* (Mead, '97); *Bdellodrilus* (Tannreuther, '15); *Thalassema* (Torrey, '03); *Lumbricus* (Kleinenberg, '79, and

Wilson, '90); Capitella (Eisig, '98); Podarke (Treadwell, '01); Arenicola and Sternopsis (Child, '00); Neretinea (Blockmann, '82); Planorbis (Holmes, '00); Unio (Lillie, '95); Argobuccinum (Philpott, '25); Physa (Wierzejski, '05); Crepidula (Conklin, '97); Dinophilus (Nelson, '04); Aplysia (Carazzi, '00); Umbrella (Heymons, '93); Sphaerium, and other forms and thus appears the most common, if not the universal, mode of origin for this germ layer. In the polyclad Discocoelis, according to Lang ('84), the mesoderm is derived from the ectomeres. Mead ('97), because of the origin and behavior of another cell in Discocoelis, believes it to be the mesoderm cell. This has not been verified for Discocoelis, although Surface ('07) finds that in Planocera the cell *4d* produces the mesoderm and all of the endoderm, since the macromeres degenerate. Mead ('97) states that the cell *M* belongs to the 'ideal' sixty-four-cell stage in Amphitrite, and believes this is true for other forms. This is not the case. In Sphaerium and in Unio, *M* is produced at about the thirty-two-cell stage, in Dinophilis and Argobuccinum at the twenty-nine-cell stage, and in Dreissensia at the forty-five-cell stage.

Regardless of the time of its production and its relative size as compared to *D*, the cell *M* always divides equally. In most cases the division that produces *M* is very unequal (compare Bdellodrilus, Dinophilus, Crepidula, Amphitrite). In Sphaerium this division is about equal. Equal division in *M* constitutes the first bilateral division in the posterior part of the embryo in all forms having spiral cleavage except in Planocera, where there is one unequal division of *4d* before equal division begins. In some forms equal division is delayed as in Dinophilus (Nelson, '04). In Sphaerium it occurs very early. Further comparisons are unnecessary. The important point is the bilateral division of *M* in all cases cited except Planocera.

The kind of division occurring in the paired mesoderm-forming cells is also strikingly similar in the various forms studied. With the exception of Clymenella, in which the first daughter cells of the pair are the same size as the parent,

a teloblastic cleavage occurs in these cells that results in the production of the mesoderm bands. These bands were thought to be ectodermal in origin by Salensky ('82) and Kleinenberg ('79 and '85). Wilson ('90) demonstrated their true origin. The number of cells produced by teloblastic division is variable. In *Sphaerium* three divisions in these cells along with division of the daughter cells results in the establishment of two irregular masses of mesoderm. The absence of definite germ bands such as occur in annelids is no doubt correlated with differences in adult form between annelids and molluscs. Division of the paired mesoderm cells may begin before or after they migrate to the interior. In *Sphaerium* the production of small mesoderm cells begins before and ends after the large cells have reached the inside. In Podarke, Treadwell ('01) finds that migration occurs before *M* and *M.1* divide.

The major portion of the mesoderm in a majority of forms is shown to be derived from the cell *d.4* (*M*) of the cleavage stages, although mesoderm of different origin is present in most species in the form of larval mesoblasts. In all cases the behavior of these two kinds of mesoderm is different. Larval mesoblasts divide irregularly and are mesenchymatous, while division in the primary mesoblasts is teloblastic and their derivatives adhere to form the germ bands. Lillie ('95) states that the larval mesoblasts provide the contractile elements used in the early stages of development. They are therefore transitory. The exact origin of the larval meso-

Fig. 35 Oblique transverse section through an early trochophore stage, showing the two germ cells on one side only. $\times 258$.

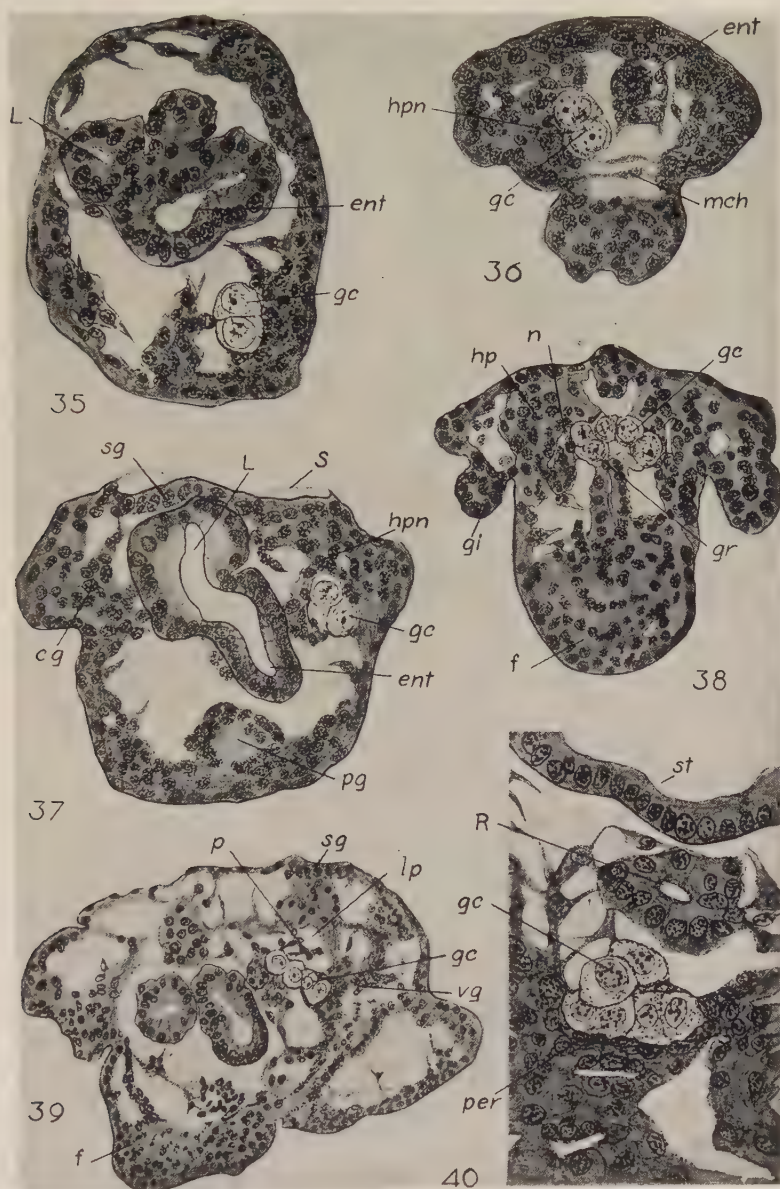
Fig. 36 Oblique transverse section of a slightly older trochophore than is shown in figure 35. The relation of the germ cells to the anlage, pericardium, and nephridium is shown. $\times 215$.

Fig. 37 Longitudinal section through an early-embryo stage, to show the relative position of the germ cells and the archenteron. $\times 215$.

Fig. 38 Transverse section of an embryo at the end of the inactive period of the germ cells. $\times 215$.

Fig. 39 Longitudinal section through an embryo, showing the formation of the right gonad. $\times 100$.

Fig. 40 The gonad region of a slightly older embryo, showing the difference in appearance of the germ cells and adjacent cells. Oblique section. $\times 430$.



Figures 35 to 40

blasts was not determined for *Sphaerium*. They probably come from cells in the anterior region of the embryo, since a few scattered cells are present in the region of the descendants of the second generation of micromeres anteriorly. None of it is formed from *M*. In *Unio*, *Sphaerium*, and *Bdellodrilus* *M* is all primary mesoderm, while in *Thalassema*, *Crepidula*, *Podarke*, *Physa*, and others it contributes in varying amount to endoderm formation also. The remainder of the mesoderm is formed in different ways in various species. In *Thalassema* the first and third generations of micromeres in the *a*, *b*, and *c* quadrants contribute; in *Unio* *a.2* is the only other cell that contributes; in *Crepidula*, *a.2*, *b.2*, and *c.2*; in *Podarke*, *a.3*, *b.3*, and *c.3*; in *Physa* *b.3* and *c.3* contribute. Further comparisons could be made, but would serve only to show that forms can be found in which almost any combination of the first, second, third, and fourth generations of micromeres contribute to the mesoderm as a whole. The exact origin of the larval mesoblasts was not determined in *Sphaerium*, because it is evident that these cells contribute nothing to the formation of germ cells.

Period of inactivity of the germ cells

Description and location. At the time of gastrulation (figs. 32 and 33) two masses of cells, in contact with the body wall and one on either side near the posterior end, constitute the major portion of the mesoderm. In each mass are two cells (fig. 33, *gc*) that have been designated as primordial germ cells, since they are structurally different from other cells and ultimately form the definitive sex cells in the gonad. Meisenheimer ('01 a, p. 426) describes the production of germ cells as the first differentiation of this mass and shows by a comparative table, including *Paludina*, *Cyclas* (*Sphaerium*), *Dreissensia*, and *Limax*, that the heart, nephridium, pericardium, and germ cells all develop from this mass of undifferentiated cells. The order of production of these structures varies in the different genera. In *Sphaerium* the order is primordial germ cells, nephridia, and lastly the heart and pericardium.

These symmetrically rounded masses of mesoderm in Sphaerium are composed in part of cells probably derived from blastomeres in the anterior part of the embryo, but mostly derived from the cell *d.4*, or *M* of the cleavage stages. Although the mesoderm cells considered collectively have a double origin, no attempt will be made to distinguish between the two kinds in the remainder of the discussion. It has been pointed out that the germ cells are derived from the primary mesoblasts *M* and *M.1*. The larval mesoblasts contribute nothing to their formation.

The four primordial germ cells (figs. 34, 35, and 36, *gc*) appear never to give rise to anything but potential germ cells and can easily be distinguished by their peculiar structure and appearance throughout later stages of development. As shown in these figures, these primordial germ cells are the largest in the embryo. Their nuclei are much larger than those of other cells and are clear except for the nucleolus and fine strands of chromatin. Their cytoplasm is somewhat clearer than that of the other cells and is smaller in proportion to the nucleus as compared with other cells. The germ cells retain this characteristic appearance during the intervening stages until they begin to increase in number as the definitive gonad is formed. There is no difficulty in recognizing them at any time.

Zeigler ('85) recognized that certain cells in this mass of mesoderm were germ cells. His figure 6 on plate XXVII shows a cell, *M.1*, that is similar to the larger cell shown in figure 32 by Stauffacher ('93). Zeigler ('85) also shows the germ cells of one side of the embryo after gastrulation in his figure 12 c, and in a later stage in his figure 19, both of plate XXVII.

Changes in location. After their first appearance in the mesoderm, the number of the primordial germ cells remains constant for a time, although their position changes. This is designated as the period of inactivity, because there is no change in appearance and because the changes in position are accomplished by factors outside the germ cells. During

this time there is rapid proliferation of cells in other regions, but the four primordial germ cells remain as two cells on each side of the embryo without further divisions until they resume their activity after reaching the place where the gonad is formed. The differentiation of the mesoderm is described by Meisenheimer ('01 a).

Four factors may conceivably play a part in this change in location: invagination; proliferation of cells in other regions and the consequent increase in size and change of shape of the embryo; movements of the embryo and of the contractile cells in particular; movements of the parent animal in which the embryo is developing. The first two factors would seem most important, the third probably would not exert any great influence in changing the location of the germ cells, while the last would be of little if any importance, since the shape of the embryo is maintained regardless of the disposition of parts in the parent.

With the ingrowth of the endoderm of the posterior midline, the two masses of mesoderm containing the germ cells are spread apart and pressed against the body wall. The subsequent elongation of the gut and the general elongation of the embryo consequent to invagination tend also to change the position of the mesodermal masses by moving them farther forward in the embryo. Proliferation of the mesodermal cells between the germ cells and the body wall carries the germ cells inward and increases the size of the remainder of the undifferentiated mesoderm (figs. 32 to 36). Rapid growth in the region of the foot and the sides of the body carries the body wall ventrally, so that the germ cells become more dorsally located (fig. 36).

At the beginning of the differentiation of the nephridia the germ cells lie on either side of the embryo at the lower margin of a mass that is forming the nephridia, but has not yet begun to form the pericardium. The nephridial masses elongate and become detached from the remainder and form the nephridial tubes, while the germ cells remain attached to the undifferentiated remainder of the mesoderm. The cavity that

now appears in the lower portion of the mesoderm above the germ cells is the lower pericardial cavity. The germ cells are attached to the ventral surface of its ventral wall. They begin division to form the cells of the definitive gonad during the formation of the upper pericardial cavity and the heart. Thus the period of inactivity is brought to an end and the final cycle of germ-cell development begins.

A detailed account of the changes occurring in the other mesoderm during the period of inactivity of the germ cells is given by Meisenheimer ('01 a) for *Sphaerium*. Zeigler ('85) recognized that the germ cells are finally separated from the lower pericardial wall, but gives a very meager description of the process.

Formation of the gonad and its regionation into male and female portions

In the early embryo. Differentiation of the kidney, pericardium, and the heart is not completed in *Sphaerium* until the latter part of the early-embryo stage. Before this time the germ cells have begun to increase in number (figs. 37 and 38). Thus there come to be two groups of germ cells attached to the ventral side of the differentiating pericardium and meeting in the midline (fig. 38). A thin layer of flattened cells surrounds the germ cells and holds them in shape as two bean-shaped, solid masses with the inner surfaces in contact. Proliferation of the germ cells is slow. Mitotic figures are rare as compared to the large number of actively dividing cells in the regions of gill formation and in the structures developing on the ventral side.

Both masses of germ cells increase in size. Since the body width increases more slowly than body length and depth, the gonads (figs. 39 to 42) find less resistance to growth longitudinally and ventrally. Elongation of the gonad occurs anteriorly until it is almost in contact with the liver, which in the meantime has been developing its secondary lobes and is extending backward toward the nephridia and gonads.

The number of cells on each side in the gonad increases from two to about twelve or fourteen during this period of the early embryo. Counts show approximately the same number on each side at any given time (figs. 42 to 44). It is possible that some of the cells were counted twice and that some were not counted at all, but the method of making camera-lucida drawings on transparent paper and then counting from these drawings is fairly accurate. The greatest variation in number on each side was two cells. Each of the new germ cells is derived from a cell like itself, already in existence, and never from somatic cells. The large size and peculiar structure of the germ cells make them easily distinguishable from the somatic cells of the peritoneum immediately surrounding the gonad. In the peritoneal cells (fig. 43, *per*) the nuclei are oval, with the greater diameter about twice the shorter. The nuclei of the germ cells are larger and spherical. This peritoneal covering of the gonad is also mesodermal in origin and its function is purely one of support. All the cells within the gonad at this time are germ cells, and even a superficial examination of cells in other regions of the body would not permit them to be mistaken for germ cells. No potential germ cells are found elsewhere in the embryo in *Sphaerium*. This differs from the condition in the vertebrates. Witschi ('21 and '22) finds indifferent cells, outside the gonad, that later undergo metamorphosis into typical germ cells. The same has been described for other forms (compare Firket, '12; Hargitt, '26, and Hann, '26).

Fig. 41 Transverse section through an older late embryo, showing the location of the gonad and its relation to other structures after the differentiation of the nephridium, pericardium, and heart. $\times 100$.

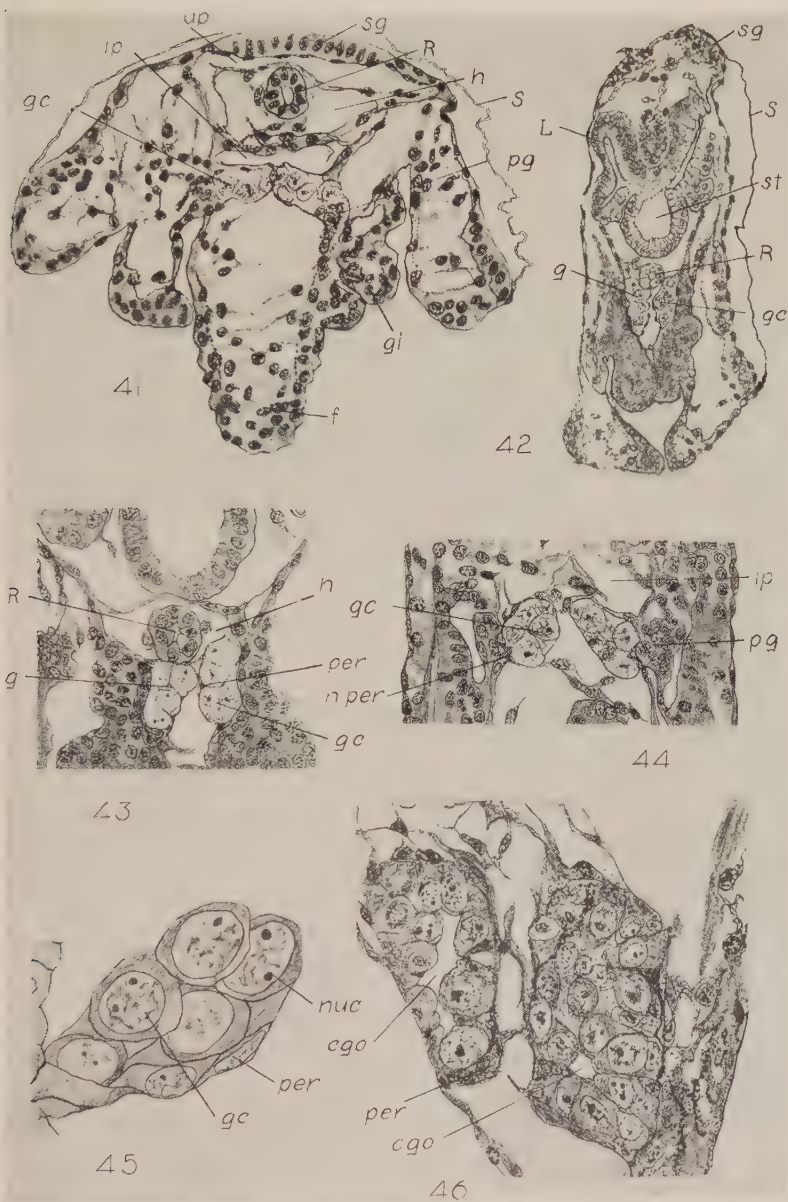
Fig. 42 Transverse section through a late-embryo stage, showing location of the gonad. $\times 50$.

Fig. 43 Region of the gonad of figure 42, under higher magnification. $\times 200$.

Fig. 44 Section of the gonad region of a slightly younger late-embryo stage than that shown in figure 43. $\times 200$.

Fig. 45 Section of the right gonad of a slightly younger embryo than that shown in figure 44. Five of the eight primordial germ cells present are shown. $\times 650$.

Fig. 46 Oblique frontal section of the gonad region of a very late-embryo stage. The cavity of the gonad is being formed. $\times 300$.



Figures 41 to 46

In the late embryo. The best distinction that can be made between the early embryo and the late embryo is to characterize the former stage as a period of organ formation, and the latter stage as one of development of already formed organs to a state comparable with their condition in the adult. The history of the gonad in the late embryo as thus defined is principally a continuation of the early embryonic history, since the gonad is being formed early in the stages preceding the late embryo and before all the other organs have been formed. Its history in the later embryonic stages is characterized chiefly by increase in the number of primordial germ cells and by changes in size and shape. The new features of this period are the formation of the ducts and the separation of the male and the female region.

During early development the gonad (figs. 42 and 45) was a solid mass of germ cells surrounded by peritoneal cells lying on either side of the body next to the place of formation of gills and pedal ganglion. At this time both sides are the same size and shape. As the length increases the anterior ends of the gonads become separated farther and farther.

The originally solid gonad on each side develops a cavity in the center (fig. 46, *cgo*) that increases in size as the gonad grows. This cavity is carried posteriorly through the wall of the gonad by a spreading apart of the adjacent cells to a point where it empties into the common cloacal chamber near the opening from the nephridium. The mass of cells posterior to the gonad increases posteriorly until the wall of the cloacal chamber is reached. The opening appears in the anterior end and proceeds slowly toward the posterior end, reaching completion about the time the embryos are extruded from the parent. The elongation of the gonad produces two short club-shaped structures with their posterior thicker portions in contact, while their anterior thinner ends spread apart slightly as they near the liver in front. Small evaginations appear in the anterior wall of each gonad as it continues to grow forward. These shallow pockets deepen and become more definitely separated from one another to form

the lobes of the male region. The male and the female regions of the gonad are closely associated in the embryo, and complete separation of these two regions does not occur until the animal is older and has begun to produce mature spermatozoa and ova. The connection between the male and female portions of the gonad is the original cavity of the gonad, and as growth proceeds this cavity becomes attenuated to form the tube that carries the male sex products to the female



Fig. 47 A portion of one lobe of the testis of a mature specimen, showing regionation of cells in maturation. $\times 215$.

Fig. 48 Longitudinal section through part of the gonad of a mature animal, to show the relation of the sperm duct of the testis and ovary.

region, from which they reach the cloacal chamber by way of the larger hermaphroditic duct. The female portion is not lobed, but consists merely of the enlarged posterior portion of the gland from which the hermaphroditic duct emerges (compare figs. 46 and 48).

Maturation

In the examination of successive stages after hatching there is no evidence of maturation in my material until the animals have reached a length of 2.5 mm. Stauffacher ('93) described the cytoplasmic growth of the oocyte in *Sphaerium*, but not the nuclear changes. I have found no account of the changes in the male sex cells during maturation. My observations confirm those of Stauffacher ('93), except that I have not observed oogonia or oocytes contributing to the storage of material in the growing oocyte, as shown in his figures 10a, 10b, and 10c of plate XI. While my material does not permit me to trace the details of meiosis, young oocytes show that the early stages are occurring during the very early growth period. The diffuse chromatin granules condense into leptotene threads (compare Winniwarter, '00), and in older cells these are condensed into rounded masses that occur in pairs. Evidently the synaptic stage occurs early during growth. The material as prepared does not permit counting of the chromosomes. These are always clumped on the spindle during the metaphase and anaphase stages, so that the number cannot be determined. It is presumed that both meiotic divisions occur after the egg is set free from the wall of the ovary, since no polar bodies are found before the eggs reach the brood pouches. I have never found either of the meiotic divisions in the egg.

In mature animals all stages of spermatogenesis are to be found in the lobed male region of the gonad throughout the year. Spermatogonia are indistinguishable from the primordial germ cells in the late-embryo stages (fig. 47, *spg*). They are peripherally located in the testis, while the first and second spermatocytes (fig. 7, *spc*) are nearer the cavity and the

spermatids (fig. 47, *sdt*) lie next to the cavity. The male region of the gonad is more favorable than the female region, but still does not show sharply defined chromosomes. Counts were made of the late prophase of the first spermatocytes and the numbers vary from sixty-eight to ninety-eight. Second spermatocyte prophases show slightly more individuality of the chromosomes, but here again counts gave varying results, ranging from thirty-two to forty-six. The chromosomes go on the spindle irregularly. Four of them always reach the equatorial position first. These four seem to have no permanent relation to the others. During the anaphase also (figs. 50 and 51) four chromosomes are always in advance of the others as they pass toward the poles. It is presumed that these result from divisions in the four that were first to arrive on the equatorial plate, although this could not be determined, since all the chromosomes are of about the same size and shape and their outlines are hazy. The chromosomes could not be counted in somatic cells of any body region. The chromosomes of all the cells are small and clumped on the mitotic spindle. This behavior of four chromosomes cannot be correlated with sex differences in the primordial germ cells, because it occurs also in somatic cells. It has been observed in the gills, the lining of the gut, cleavage stages, brood-pouch cells with multipolar spindles, and elsewhere in the animal (compare figs. 50 to 53).

The first meiotic division seemed to be reductional, at least for the majority of the tetrads. Secondary spermatocytes occur in pairs in the prophase and each contains approximately half the number of chromosomes present in the prophase of the first spermatocyte. In the late telophase of the second meiotic division the chromosomes are closely clumped at the ends of the spindle. When the two cells separate, spermioteleosis begins (fig. 47). The spermatids always occur in quartets (fig. 47, *sdt*) immediately after the second meiotic division. The periphery of the early spermatid nucleus stains deeply, while the central portion is clearer and stains faintly. Very little cytoplasm is present. The material was not fixed

to demonstrate the Golgi bodies, hence no statement can be made concerning their behavior. No visible structures can be interpreted as acroblasts or acrosomes, either in the early or late stages of spermiogenesis. Very fine granular mitochondria are present. These become condensed in the elongating spermatid to form a nebenkern consisting of a variable number (two to six) of relatively large granules that stain with acid fuchsin. Their history was not followed. Centrioles are not visible in either spermatids or spermatozoa, although they are evident during the metaphase and early anaphase of mitosis. The mature spermatozoon is fusiform, with an elongated head.¹ The middle piece shows a deeply staining area adjacent to the head, while a clear area containing the nebenkern is found between this and the tail. The tail is very delicate and is seen only in material that has been counterstained. The details of spermiogenesis could not be followed accurately enough to permit further description or presentation of figures.

There is no indication in my material that indifferent cells of the gonad differentiate into sex cells, as described by Witschi ('21) and Swingle ('21) for frogs and by Hargitt ('24 and '25) for the salamander and rat. The germ cells in *Sphaerium* seem to be continuous from their original seg-

Fig. 49 Diagram of an embryo in the hatching stage, to show the relation of most of the structures in the body, made from a whole mount of an embryo dissected from the gills. $\times 50$.

Fig. 50 Oblique section through a twelve-cell stage in which one micromere shows a quadripolar spindle. $\times 35$.

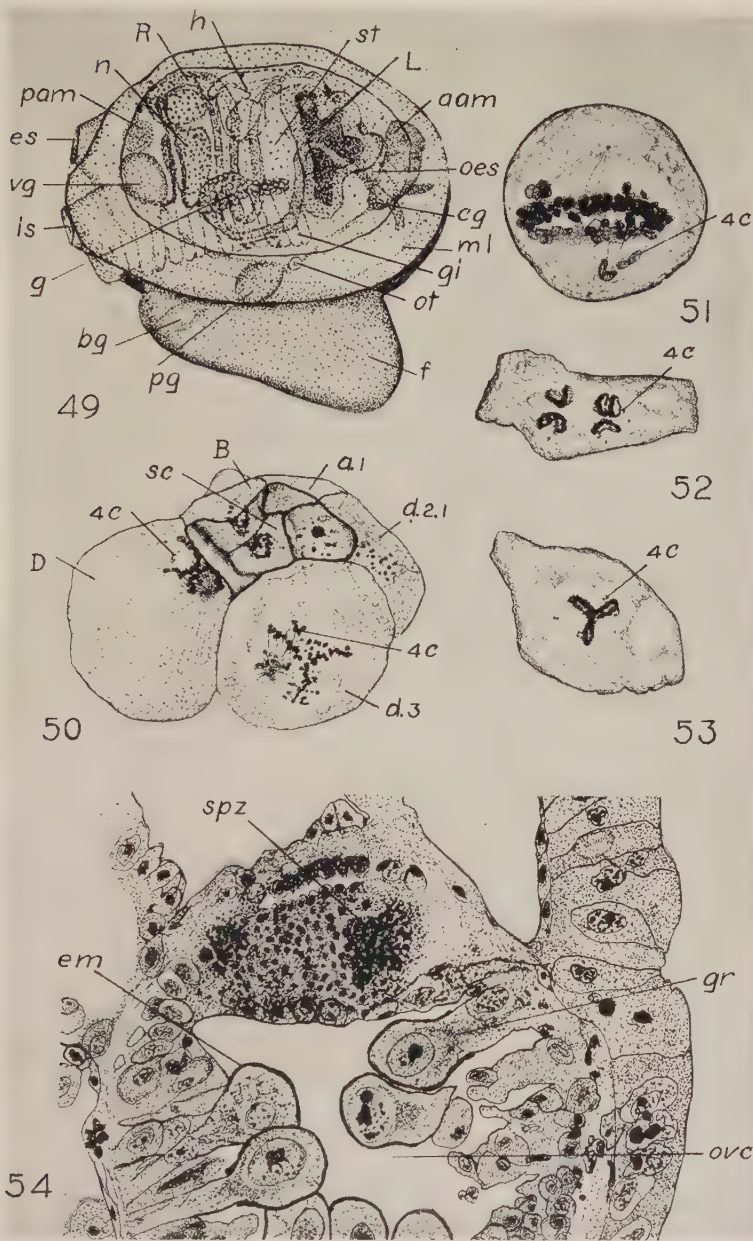
Fig. 51 Secondary spermatocyte in early anaphase, showing four masses of chromatin (4C) which precede the remainder in passage to either pole. $\times 1200$.

Fig. 52 A multipolar cell in the nephridium, showing the four chromatin masses that precede the remainder in passage to either pole. $\times 600$.

Fig. 53 A tripolar spindle in a brood-pouch cell, showing same chromatin behavior as figures 51 and 52. $\times 600$.

Fig. 54 A portion of the gonad of a recently matured specimen, showing close relation of male and female regions of the gonad. $\times 275$.

¹ *Sphaerium striatinum* from the vicinity of Columbia, Missouri, produce spermatozoa with heads approximately 50 per cent longer than sperm of specimens collected at Woods Hole, Massachusetts, although these were identified as belonging to the same species (compare p. 546).



Figures 49 to 54

regation during cleavage to the time of maturation. I have found no evidence of primordial germ cells giving rise to anything except functional germ cells. It is possible that some primordial germ cells are lost as a result of the method by which nutritive cells become incorporated into the cytoplasm of the oocytes. Such cases are described by Stauffer ('93), although I have not observed them in my material. Whole cells adjacent to a growing oocyte do become incorporated into the oocyte cytoplasm, but so far as I can determine, these nutritive cells are epithelial cells and not primordial germ cells. As previously described, these two types differ greatly in structure. There is no indication of a precocious maturation of the germ cells in *Sphaerium* as described by Witschi ('21) and others. Once maturation begins, it is apparently a continuous process during the life of the individual, though the rate may vary seasonally. Spermatozoa produced by very young animals are in no way different from those in older specimens except that the total number is smaller because the gonad is smaller. The same is true of the ova. No difference can be seen in ova of young and full-grown specimens of *Sphaerium*. Maturation begins only after the young have been set free from the gills and have attained a length of about 2.5 mm. Gilmore ('17) states that in *Sphaerium simile* young are not produced until the animals reach a length of 10 mm., though the gonads are fully formed in individuals 6, 7, and 8 mm. in length. This differs from *Sphaerium striatinum*, in which young are present in the brood pouches of 3-mm. specimens and are set free from specimens of 4 and 5 mm. in length.

No clues have been obtained suggesting the factors that determine which cells of the gonad will become spermatozoa and which will become ova. The cytological methods used reveal no differences in the primordial germ cells that forecast later differentiation into either ova or spermatozoa. The work of McClung ('02), Montgomery ('04), Stevens ('08), Harvey ('16), Carothers ('17 and '21), and many others has demonstrated the correlation between sex and specific chro-

mosomes. I have found no description of such differences for monoecious animals. Schrader ('21 and '28), Bridges ('21, '22, and '25), and others show that environmental factors are in part responsible for sex or that it is a matter of the relative quantitative action of sex-determining factors not definitely located in a single pair of homologous chromosomes. The relations of sex to chromosomes in Mollusca seem to have been investigated only in some Gastropoda (compare Schrader, '28, pp. 67-71).

Discussion

Primary cellular differentiation or segregation of the primordial germ cells has been described in representatives from many groups of animals. Evidence has been presented to show that this is the general occurrence, although exceptions can be found. In forms that show an early segregation of primordial germ cells, whether these cells later give rise to definitive germ cells or not, there follows a period of inactivity. Hegner ('14, p. 376) characterizes this period of inactivity as one of separation of the germ cells into two groups, cessation of cell division in the primordial germ cells, and active or passive changes in position before the formation of definitive sex cells. This period of inactivity occurs during the growth of the embryo and continues until the larval stage is almost attained.

In claiming that early segregation of germinal material occurs in *Sphaerium*, it is not my intention to imply any activity on the part of this material during segregation or any continuity of unchanged egg substance that is responsible for the production of germ cells. It is my belief that cleavage and mesoderm formation in *Sphaerium* result in the separation of other potentialities of development from that of producing germ cells. There is no evidence of activity on the part of the primordial germ cells until they have reached their place of development in the gonad.

According to Hargitt ('25), the production of germ cells in vertebrates is a matter of cellular differentiation compar-

able with that of somatic cells, and not one of early ontogenetic segregation. The case in *Sphaerium* agrees with such an interpretation, except in one respect: while other types of cells differentiate from embryonic cells early in ontogeny, the germ cells are inactive and retain their embryonic condition until they differentiate into functional germ cells in the adult. From this viewpoint functional germ cells are not to be considered as the first cells in which differentiation occurs in the developing organism, but as the last cells to reach a functional state.

In *Sphaerium* there is no evidence that somatic cells ever give rise to germ cells. If a cell is specialized for the performance of some bodily function, it seems incapable, in *Sphaerium* at least, of giving rise to a new individual. Germ cells are the only cells having this capacity. The end result of meiosis is comparable to differentiation of somatic cells in that functional cells are produced. In *Sphaerium*, so far as can be determined, all functional germ cells are the lineal descendants of primordial cells that have been 'left behind,' as it were, while cellular differentiation was occurring in other parts of the body. That these primordial germ cells have lost all their capacities for differentiation, except that for the production of gametes, is shown by the fact that they seem never to give rise to any type of cell except gametes.

In cases where so-called dedifferentiation of somatic cells is described (Gatenby, '16; Kingery, '17; Spehl and Polus, '12, etc.), it is possible to interpret these changes in terms of cells that have retained capacities for differentiation in more than one direction. Under a given set of developmental conditions one of these potentialities has been expressed in beginning differentiation toward a particular type of cell, but only to a limited extent. If differentiation has not been completed, changing the environmental conditions of the cell may make possible the expression of an entirely different capacity for differentiation. The cell may complete differentiation in this new direction and lose the characteristics it had acquired temporarily. The evidence is inadequate to

show that a highly specialized cell, like a muscle or a nerve cell, having a limited function can become dedifferentiated and that the resultant undifferentiated cell can redifferentiate into an entirely different type of cell with different function, although such cases have been described (compare Vandel, '22, p. 428). Until individual cells have been followed through the process of dedifferentiation and redifferentiation such evidence will not be conclusive. The changes described as dedifferentiation may as well be interpreted as the degenerative changes preceding death. In other cases, like that of the planarians just cited, it is difficult to rule out the possibility that 'formative cells' (compare Curtis, '02 and '28) have not replaced those that seem to have arisen by dedifferentiation. The great body of evidence indicates that complete cell specialization is irreversible. It is true, however, that cells do retain more than one potentiality for complete specialization if they are not too highly specialized. Germinal epithelium cells belong to this group in the vertebrates. They may become peritoneal cells, germ cells, Sertoli cells, etc., as shown by many workers. In *Sphaerium* the only source of germ cells is the primordial germ cells.

Production of functional germ cells is, then, a matter of cell differentiation and specialization, comparable in a sense with tissue differentiation in other types of cells, but differing in that it is delayed. The so-called early segregation of germ cells is merely suspended activity and delayed differentiation. Although we know little of the causes of differentiation, we do know the time of differentiation in many cases. The other tissues in *Sphaerium* differentiate, so that the undifferentiated line of primordial germ cells is conspicuous early in ontogeny. That no continuous line of germ cells is evident in some vertebrates is not to be denied. The question is whether the germinal material is not only lost to view, but is lost entirely and appears *de novo* from some specialized body cell. It may be true that the potentiality for producing germ cells exists, perhaps, in several kinds of cells, and that the particular set of conditions necessary for the expression

of this potentiality occurs only in a limited portion of the body, the gonads, and only at certain cyclic periods in the life of the organism. Gatenby ('23) thinks this is true for Amphibia. Germinal continuity of some sort seems to exist, as evidenced by the fact that at certain periods cells become differentiated for reproduction. Whether cells carrying such potentialities for specialization can be recognized early or late in ontogeny seems of little consequence. Their behavior in the process of maturation and fertilization and in subsequent development into a new individual is strikingly similar in their fundamental events in all multicellular animals.

The claim that egg cytoplasm is passed unchanged through a continuous line of cells that will differentiate into germ cells cannot be substantiated. Development is a continuous process of change. Cleavage separates the cytoplasm of the egg into portions whose potentialities for normal development are different. Every change that occurs in development affects the materials in the cells. The passage of the cytoplasm of a particular cell, unchanged, to the descendants of that cell is not in keeping with our concept of protoplasm existing in a state of dynamic equilibrium.

The zygote receives from each parent certain capacities for development. As development proceeds new capacities are acquired. In addition to these potentialities, the environmental factors determine the nature and extent of expression of the capacities inherited in the zygote and acquired in development. In view of these facts, it is almost inconceivable that germinal material remains constant in substance or behavior throughout its history in one generation. Egg cytoplasm possesses certain capacities for interaction with the nucleus that result in cellular differentiation. One of these is the capacity to produce germ cells under normal conditions at a definite time and place in the organism. The time varies greatly, just as the time of differentiation of other types of cells varies in different animals. That a particular region can be recognized as the part from which germ cells ultimately will develop does not imply that this cytoplasm re-

mains unchanged throughout subsequent cellular generations. In *Sphaerium* the inherent capacity for differentiation into germ cells descends through a particular line of cells to the primordial germ cells.

In many cases germinal continuity has been claimed, but is incompletely demonstrated. In other cases discontinuity has been claimed. The question of germinal continuity, therefore, remains unsettled. Direct cellular continuity can be demonstrated in some animals, *Ascaris*, *Miastor*, and other insects, *Sphaerium*, etc., but is believed not to exist in others like certain *Amphibia*, *Mammalia*, etc. If germinal continuity exists in all animals, it must be similar to the sort of continuity postulated for heritable somatic characteristics. The argument from facts to conclusion supporting the theory of germinal continuity is of the same nature as that supporting the gene theory in heredity and the atomic theory in chemistry. Both the gene theory and the atomic theory have undergone as much modification at the hands of investigators since their first promulgation as the original theory of germinal continuity of Weismann. It cannot be maintained in all cases that primordial germ cells are recognizably different from other embryonic cells or that they are present before gonad formation. The theory that 'germinal continuity' exists because something in the life cycle of animals behaves as if it were continuous is open to criticism. The gene theory, 'crossing-over,' and the atomic theory can be criticized for the same reason. I shall not discuss the vitalistic aspects of these theories. Considered as working hypotheses, the value of these theories has been demonstrated by the history of their biological accomplishment. Such hypotheses have undergone much modification in the past and no doubt will be further modified as new facts become known.

SUMMARY AND CONCLUSIONS

The history of the germ cells in *Sphaerium* is traceable from the fertilized egg to sexual maturity in the new individual, making a complete cycle.

By cell lineage it can be shown that the germ cells are derived from the cell *D*, of the four-cell stage, and are definitely set aside as primordial germ cells by the third division of the paired mesoderm cells, *M* and *M.1*, arising from the single cell *M*, which in turn is the fourth micromere produced by the cell *D*.

After the formation of the mesoderm, the subsequent history of these primordial germ cells can be determined with no great difficulty, since they differ in size and appearance from other cells of the embryo.

Following a single division of the two original right and left germ cells, the two primordial germ cells on each side of the embryo remain inactive until after the differentiation of the nephridia and the beginning of differentiation of the pericardial cavities. During this period their position is changed by the development of other structures.

At the end of the inactive period mitotic division is resumed by the primordial germ cells, and this marks the beginning of gonad formation. Observations on gonad formation in this genus confirm those of earlier investigators.

Maturation is first evident in the female portion of the gonad. Cytoplasmic growth occurs and there is meager evidence of meiotic changes in the nuclei of cells in this region. Maturation begins in the male portion before full-grown oocytes are present in the ovary.

There is no indication of alternating activity in the male and female regions of the gonad in this species, since mature individuals always show male and female sex cells in all stages of development throughout the year.

The primordial germ cells, which are visibly different from somatic cells early in development, do not lose their identity at any later period. Only two kinds of cells are present in the gonad: primordial germ cells and peritoneal cells. New germ cells arise only from pre-existing germ cells and primordial germ cells apparently do not transform into somatic cells in the gonad.

The germ-cell cycle in *Sphaerium* may be divided into five periods with well-defined limits:

1. The original appearance, with the formation of two and then four primordial germ cells during the cleavage stages.

2. An inactive period, during which the two primordial germ cells on either side of the embryo do not divide, but are carried to the place of gonad formation by growth in other regions.

3. A multiplication period in the developing gonads continuing throughout the life of the animal and producing an indefinite number of primordial germ cells.

4. Maturation, including oogonial and spermatogonial growth, and the meiotic nuclear changes.

5. Fertilization.

The details of maturation and the chromosomal number could not be determined, because the chromosomes are small and clumped together.

No indication could be found of chromosomal differences in the primordial germ cells that forecast which cells would become ova and which spermatozoa. No theories are offered to explain this differentiation.

Although a definite cellular continuity for the capacity to produce functional germ cells seems demonstrable in *Sphaerium*, this does not imply that egg cytoplasm passes unchanged through the cleavage stages to the primordial germ cells.

It is believed that the characteristics by which the primordial germ cells are recognizable early in cleavage arise because these cells retain the capacity for further differentiation, in contrast with somatic cells which lose this capacity as they differentiate. Thus the production of functional germ cells is thought to be a process of differentiation comparable with that which occurs in other cells at a much earlier stage of development. In other respects the germ-cell differentiation is comparable to differentiation of somatic cells.

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